

Generation of Reactive Species and Fate of Thiols During Peroxidase-Catalyzed Metabolic Activation of Aromatic Amines and Phenols

by David Ross* and Peter Moldeust†

The horseradish peroxidase (HRP)-catalyzed oxidation of *p*-phenetidine and acetaminophen was investigated. Studies using the spin probe 2-ethyl-1-hydroxy-2,5,5-trimethyl-3-oxazolidine (OXANOH) suggested these oxidations involve the generation of substrate-derived free radicals. This was confirmed by using glutathione (GSH) in these incubations in the presence of the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). DMPO-glutathionyl radical adducts were observed using EPR spectroscopy during HRP-catalyzed oxidation of both *p*-phenetidine and acetaminophen. Investigations of oxygen uptake and oxidized glutathione (GSSG) formation during HRP-catalyzed oxidations of *p*-phenetidine and acetaminophen suggested that further reactions of the glutathionyl radical involve glutathione peroxysulfenyl radical and glutathione sulfenyl hydroperoxide production. Quinonoid products of the peroxidatic oxidations of *p*-phenetidine and acetaminophen, and their interaction with GSH via both conjugation and redox mechanisms are described. The relevance of these reactions of GSH with reactive species as detoxification mechanisms is discussed.

Introduction

Aromatic amines and phenols are widely used in industrial processes and as drugs and are therefore compounds of toxicological interest. Both of these groups of compounds act as efficient cosubstrates of various peroxidases such as prostaglandin synthase (PGS) and horseradish peroxidase (HRP)(1,2). We have been investigating the metabolism of the analgesic drug acetaminophen (*N*-acetyl-*p*-aminophenol) and the aromatic amine *p*-phenetidine (4-ethoxyaniline), both of which are metabolites of the analgesic phenacetin. Phenacetin may induce analgesic nephropathy after chronic dosage and has also been shown to induce kidney carcinoma (3,4). As the kidney is rich in peroxidases such as PGS (5) then peroxidatic metabolism of the two primary phenacetin metabolites, acetaminophen and *p*-phenetidine (6), in the kidney may contribute to phenacetin-induced nephrotoxicity.

In order to assess the toxicological implications of

peroxidatic oxidation of *p*-phenetidine and acetaminophen we have used HRP as a model peroxidase. We describe here some mechanistic aspects of our work on the HRP-catalyzed metabolism of both *p*-phenetidine and acetaminophen. In addition, on a more general basis, we have used these two compounds as model substrates to investigate the interaction of substrate-derived reactive products with the endogenous tripeptide glutathione (GSH), a thiol ubiquitous in mammalian systems which is generally considered to contribute to the detoxification of reactive species (7).

Results and Discussion

The substrate for HRP is hydrogen peroxide (H₂O₂), which oxidizes the enzyme from its Fe³⁺ resting state to an Fe⁵⁺ form (compound 1), and regeneration of the resting state of the enzyme usually occurs via two successive one-electron reductions utilizing a variety of hydrogen donors.(1). Amines and phenols can serve as reducing cosubstrates for the oxidized form of HRP and are thus oxidized to free-radical forms during this process (8-10). We were unable to demonstrate the generation of free-radical intermediates during HRP-cat-

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alyzed oxidation of *p*-phenetidine or acetaminophen using EPR spectroscopy in the presence or absence of the spin-trapping agent 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). That free-radical species were produced in these reactions was suggested by use of the spin probe 2-ethyl-1-hydroxy-2,5,5-trimethyl-3-oxazolidine (OXANOH). This compound can be oxidized by other free radicals to generate the radical form of the spin probe -OXANO \cdot , a relatively stable nitroxide which can be observed easily by using EPR spectroscopy (11). The inclusion of OXANOH during HRP-catalyzed oxidation of acetaminophen led to the generation of an EPR signal indicative of OXANO \cdot production (Fig. 1). The peroxide-, enzyme-, and acetaminophen-dependence of the EPR signal confirmed that the species responsible for the oxidation of the hydroxylamine was a product of enzymatic oxidation of acetaminophen (12). Similar results were obtained by using *p*-phenetidine in place of acetaminophen (13).

One of the cells' protective mechanisms against reactive species involves the thiol GSH, which is known to detoxify electrophiles via the formation of conjugates which are subsequently excreted from the cell (7). There are few data, however, concerning the interaction of GSH with free radicals generated during the metabolism of xenobiotics. The inclusion of GSH during the HRP-catalyzed oxidation of *p*-phenetidine led to a decrease in the removal of the aromatic amine, the extent of which was dependent on GSH concentration (13). This suggested that GSH may reduce the amine radical back to the parent substrate, a process which would involve the generation of a glutathione radical. Thiyl radicals have been observed previously by using EPR spectroscopy in conjunction with the spin-trap DMPO (14,15), and we employed this technique to investigate

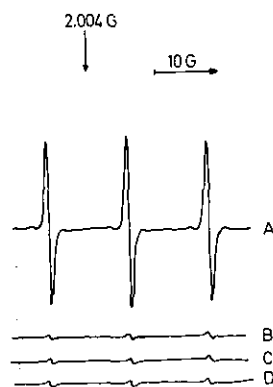


FIGURE 1. EPR spectra obtained during HRP-catalyzed metabolism of acetaminophen in the presence of OXANOH: (A) acetaminophen (1 mM), HRP (0.2 μ g/mL), H_2O_2 (0.25 mM), OXANOH (1 mM); (B) as for (A) but minus acetaminophen; (C) as for (A) but minus HRP; (D) as for (A) but minus H_2O_2 . Instrumental conditions: receiver gain = 4×10^8 , microwave power = 10 mW, modulation amplitude = 0.2 G, time constant = 0.3 sec, scan time = 2 min. Reactions were started by addition of peroxide and spectra recorded after 1 min.

their formation under these conditions. The inclusion of GSH and DMPO in an incubation containing HRP, H_2O_2 and *p*-phenetidine led to the production of a paramagnetic signal (Fig. 2A) ($a^N = 15.0$ G, $a^H = 16.3$ G) consistent with the generation of a DMPO-glutathionyl radical adduct (14,15). Such a signal was not observed in the absence of thiol, enzyme or *p*-phenetidine (Fig. 2B-D). When acetaminophen was used as cosubstrate in the presence of GSH, DMPO, HRP, and H_2O_2 , the spectrum shown in Figure 2E was observed (12); it is essentially identical to that observed with *p*-phenetidine. These results show that substrate-derived radicals are produced during HRP-catalyzed oxidation of *p*-phenetidine and acetaminophen. This has recently been confirmed in the case of acetaminophen, where the phenoxy radical has been observed directly by using a fast-flow EPR technique (16). Furthermore these data show that amine and phenol-derived radicals may be reduced by thiols such as GSH to generate thiyl radicals. This reaction represents a potential detoxification reaction—a mechanism whereby reactive, short-lived amine and phenoxy radicals can be removed from the system. There is however another radical species generated in these reactions—the thiyl radical, and whether or not the removal of amine or phenoxy radicals by GSH is

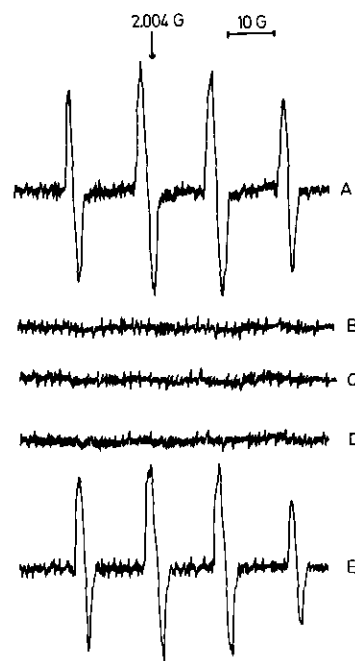


FIGURE 2. EPR spectra obtained during HRP-catalyzed metabolism of *p*-phenetidine and acetaminophen in the presence of GSH and DMPO: (A) *p*-phenetidine (0.5 mM), HRP (0.2 μ g/mL), H_2O_2 (0.25 mM), GSH (5 mM), DMPO (100 mM); (B) as for (A) but minus GSH; (C) as for (A) but minus HRP; (D) as for (A) but minus *p*-phenetidine; (E) acetaminophen (1 mM), HRP (0.2 μ g/mL), H_2O_2 (0.25 mM), GSH (5 mM), DMPO (100 mM). Instrumental conditions: as in Fig. 1, but receiver gain = 2×10^4 . Reactions were started by the addition of peroxide and spectra recorded after 1 min.

indeed a true detoxification reaction depends on the subsequent fate of the glutathionyl radical.

Thiyl radicals are known to dimerize to form oxidized glutathione (GSSG) and to interact with oxygen (17,18). During the HRP-catalyzed oxidation of either *p*-phenetidine or acetaminophen we observed increased GSSG formation relative to controls without the phenol or the amine and a GSH-dependent oxygen uptake (13,19,20) (Table 1). The interaction of the glutathionyl radical with oxygen generates the peroxysulfenyl radical (GSOO \cdot) which may then undergo a number of reactions (17,18). One possible fate of this radical is the generation of glutathione sulfonic acid (GSO $_3$ H) (21), but we were unable to detect this in our incubations. The glutathione peroxysulfenyl radical may also interact with GSH regenerating the thiyl radical which could once more interact with oxygen. Thus extensive oxygen uptake would be observed during HRP-catalyzed oxidation of either *p*-phenetidine or acetaminophen in the presence of excess thiol. That this was the case is shown in Figure 3. Utilizing *p*-phenetidine and acetaminophen concentrations of 0.5 mM and 0.25 mM, respectively, in these reactions, maximal regeneration of substrate in the presence of GSH, and therefore maximal oxygen uptake, should occur at equimolar thiol concentration, assuming that GSH interacts with a single amine or phenoxy radical. Oxygen uptake observed during HRP-catalyzed oxidation of *p*-phenetidine or acetaminophen, however, increased with increasing concentrations of GSH up to 5 mM (Fig. 3). This suggests that an oxidized glutathione radical, such as a peroxysulfenyl radical, may interact with GSH, thus causing a cyclical reaction in the presence of excess thiol leading to extensive oxygen uptake. On the basis of these results we have proposed the scheme shown in Figure 4 to explain the interaction of GSH with amine and phenoxy radicals and the subsequent fate of the glutathionyl radical.

Table 1. Formation of GSSG and oxygen uptake during HRP-catalyzed oxidation of *p*-phenetidine and acetaminophen in the presence of GSH.^a

	GSSG formation, μ M ^b	O $_2$ uptake, μ M ^c
<i>p</i> -Phenetidine ^d	440	180
Acetaminophen ^e	130	113

^a Reactions were performed at 25°C for 5 min in the case of GSSG determination and until uptake was complete in the case of oxygen uptake.

^b After subtraction of control values obtained in incubations minus *p*-phenetidine or acetaminophen.

^c No uptake was observed minus *p*-phenetidine or acetaminophen, minus GSH, minus H $_2$ O $_2$. A small oxygen uptake was observed minus HRP and the results are corrected for this.

^d Substrate 0.5 mM; HRP, 0.2 μ g/mL; H $_2$ O $_2$, 0.25 mM; GSH, 5 mM.

^e Substrate, 0.5 mM; HRP, 25 μ g/mL; H $_2$ O $_2$, 0.25 mM; GSH, 5 mM.

rearrangement to glutathione sulfinic acid (GSO $_2$ H) or hydrolysis eventually leading to the generation of GSSG are two possible pathways (Fig. 4). The toxicological relevance of the further reactions of the glutathionyl

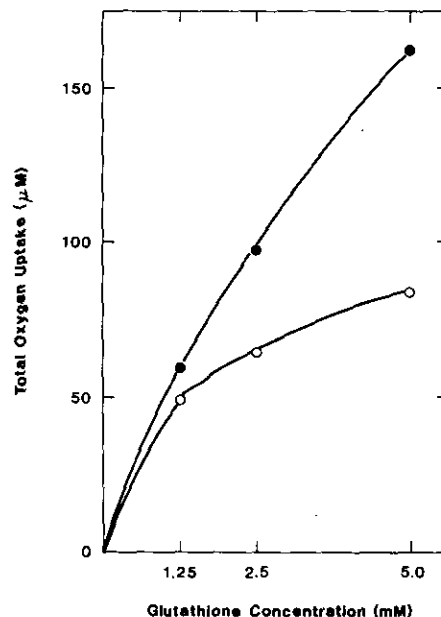


FIGURE 3. Oxygen uptake observed during HRP-catalyzed oxidation of (●) *p*-phenetidine and (○) acetaminophen in the presence of various concentrations of GSH. First for *p*-phenetidine oxidation: Conditions: HRP (0.2 μ g/mL), H $_2$ O $_2$ (0.25 mM), substrate (0.5 mM); conditions for acetaminophen oxidation: HRP (25 μ g/mL), H $_2$ O $_2$ (0.125 mM), substrate (0.25 mM).

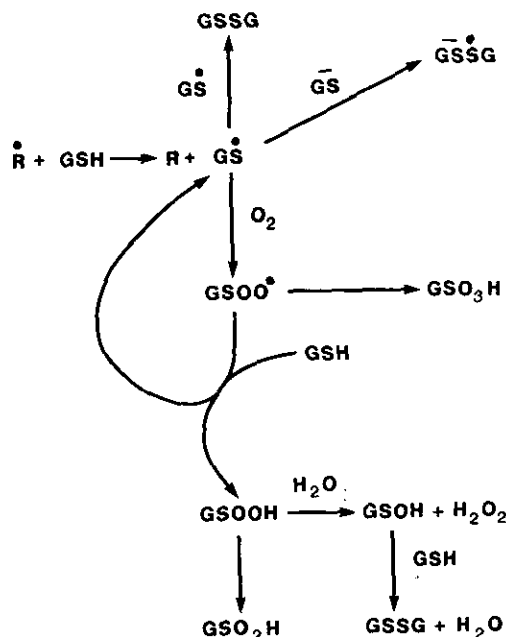


FIGURE 4. Proposed scheme for the interaction of GSH with amine and phenoxy radicals (R $^\bullet$) and the subsequent fate of the glutathionyl radical.

radical remains to be established, and therefore the question whether the interaction of GSH with amine or phenoxy radicals represents a true detoxification reaction must remain open.

Peroxidase-catalyzed oxidation of amines and phenols usually generates a variety of products due to radical-coupling reactions and the oxidations of *p*-phenetidine and acetaminophen by HRP are not exceptions to this. In the case of *p*-phenetidine, many intensely colored products are formed during HRP-catalyzed oxidation (13,24). Some of these species were capable of forming conjugates with GSH, shown by the presence of water-soluble radioactivity after extraction when GSH was added to incubations containing (14 C)-*p*-phenetidine, HRP, and H_2O_2 (25). When the conjugates formed were analyzed by HPLC, different conjugates were observed, dependent on the time of addition of GSH (26) (Fig. 5). This indicates the sequential production of a number of different reactive species during HRP-catalyzed oxidation of *p*-phenetidine. Two of the conjugates

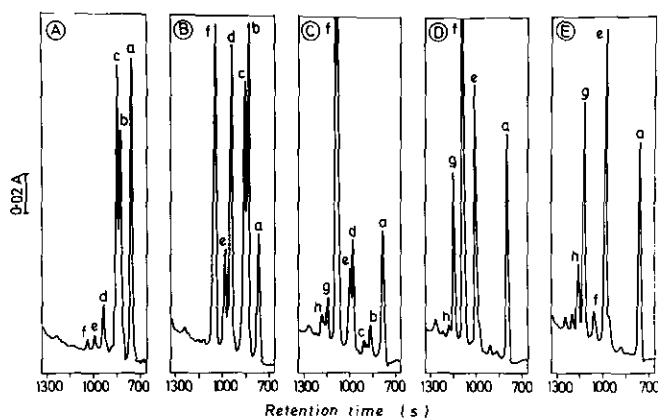


FIGURE 5. HPLC elution profiles obtained after the addition of GSH at various times to an incubation containing HRP (0.2 μ g/mL), H_2O_2 (1 mM), and *p*-phenetidine (0.5 mM). GSH (1 mM) was added (A) 1 min, (B) 5 min, (C) 30 min, (D) 60 min, and (E) 240 min after initiation of the reaction. Analytical conditions as described elsewhere (26).

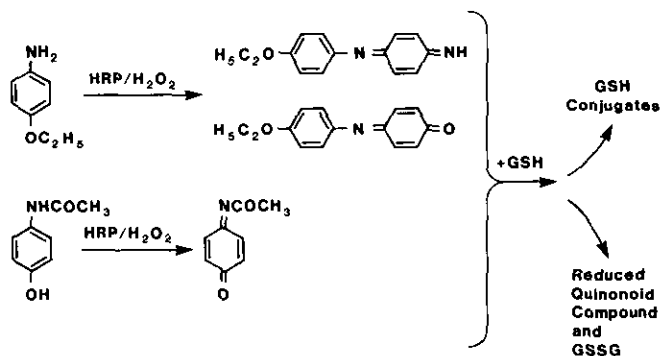


FIGURE 6. Proposed scheme showing the interaction of quinonoid reactive species produced during HRP-catalyzed oxidation of either *p*-phenetidine or acetaminophen with GSH.

were characterized by using fast atom bombardment mass spectrometry as mono-GSH adducts of 4-ethoxyphenyl-*p*-benzoquinoneimine and its diimine precursor (26) (Fig. 6A). The identity of the other conjugates formed during HRP-catalyzed oxidation of *p*-phenetidine is unknown, but double-labeling experiments using purified (14 C)-4-ethoxyphenyl-*p*-benzoquinoneimine and (3 H)-GSH have suggested that di-GSH conjugates of the quinoneimine may also be formed (26). Furthermore the conjugates isolated may exist in both oxidized and reduced forms and can be readily interconverted by redox processes. Indeed, this type of redox reaction occurs to a limited extent during the reaction of the quinoneimine itself and GSH, as GSSG is generated during this reaction (unpublished) showing that GSH may serve as a reductant towards the quinoneimine (Fig. 6A).

Peroxidase-catalyzed oxidation of acetaminophen also leads to the generation of reactive species which will form conjugates with GSH. The major species responsible for such conjugation reactions, at least formed during PGS catalyzed oxidation, has been suggested to be *N*-acetyl-*p*-benzoquinoneimine (NAPQI) (27), (Fig. 6B). As in the case of the quinoneimine derived from *p*-phenetidine, GSH also serves as a reductant towards NAPQI, but in this case the redox reaction is more significant, particularly in the presence of excess GSH (28,29). The reduction of quinonoid compounds by GSH may be toxicologically relevant, as the resultant hydroquinones would be expected to be conjugated with either glucuronide or sulfate and excreted.

In summary, we have provided evidence for the generation of free radicals and reactive quinonoid species during HRP-catalyzed oxidation of *p*-phenetidine and acetaminophen. We have also investigated the interaction of these species with the endogenous thiol GSH. GSH reduces substrate-derived radicals to regenerate substrate and produce glutathionyl radicals, whereas the thiol may interact with quinonoid compounds in at least two different ways, as a reductant or as a conjugating species.

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